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CERTIFICATE

I, Dr. Thomas Besier, Oberer Dorfgraben 71A, D-55130 Mainz, Germany, am conversant with the English and German languages and am a competent translator thereof.

To the best knowledge and belief, the attached English text is a true and correct translation of the German document titled "Ester von Polysaccharid Aldonsäuren, Verfahren zu ihrer Herstellung und Verwendung zur Kopplung an pharmazeutische Wirkstoffe" (DE 102 56 558 A1).

Signed this 10th day of August 2010

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Dr. Thomas Besier

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Translation of DE 102 56 558 A1

Ester of polysaccharide aldonic acids, methods for their production and use for coupling to pharmaceutical active ingredients

Description

[0001] The conjugation of pharmaceutical active ingredients in particular of proteins with polyethylene glycol derivatives ("PEGylation") or polysaccharides such as dextrans or, in particular, hydroxyethyl starch ("HESylation") has gained importance in recent years with the increase in pharmaceutical proteins from biotechnology research.

[0002] The biological half-life of such proteins is often too short but can be prolonged specifically by coupling to the abovementioned polymeric compounds such as PEG or HES. However, the coupling may also have a beneficial influence on the antigenic properties of proteins. In the case of other pharmaceutical active ingredients it is possible considerably to increase the solubility in water by the coupling.

[0003] The HES is the hydroxyethylated derivative of amylopectin which is the glucose polymer which constitutes more than 95 % of waxy corn starch. Amylopectin consists of glucose units which are present in α-1,4-glycosidic linkages and have α-1,6-glycosidic branches. HES has advantageous rheological properties and is currently used clinically as volume replacement agent and for hemodilution therapy (Sommermeyer et al., Krankenhauspharmazie, Vol. 8 (8, 1987) pages 271-278 and Weidler et al., Arzneimittelforschung/Drug Res., 41, (1991) pages 494-498).

[0004] HES is characterized essentially via the weight average molecular weight Mw, the number average molecular weight Mn, the molecular weight distribution and the substitution level. Substitution with hydroxyethyl groups in ether linkage is in this case possible at carbon atoms 2, 3 and 6 of the anhydroglucose units. The substitution level can in this connection be described as DS ("degree of substitution") which is based on the substituted glucose molecules as a proportion of all the glucose units, or as MS ("molar substitution") which refers to the average number of hydroxyethyl groups per glucose unit.

[0005] DE 196 28 705 and DE 101 29 369 describe possible methods for carrying out the coupling of hydroxyethyl starch in anhydrous dimethyl sulfoxide (DMSO) via the

corresponding aldonolactone of hydroxyethyl starch with free amino groups of hemoglobin and amphotericin B, respectively.

[0006] Since it is often not possible to use anhydrous, aprotic solvents specifically in the case of proteins, either for solubility reasons or else on the grounds of denaturation of the proteins, coupling methods with HES in an aqueous medium are also available. For example, coupling of hydroxyethyl starch which has been selectively oxidized at the reducing end of the chain to the aldonic acid is possible through the mediation of water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide) (PCT/EP 02/02928). However, the use of carbodiimides is very often associated with disadvantages, because carbodiimides very frequently cause inter- or intramolecular crosslinking reactions of the proteins as side reactions.

[0007] In the case of compounds containing phosphate groups, such as nucleic acids, the coupling is often impossible because the phosphate groups may likewise react with EDC (S. S. Wong, Chemistry of Protein Conjugation and Cross-Linking, CRC-Press, Boca Raton, London, New York, Washington D.C., 1993, page 199).

[0008] Therefore, the object exited to find such activated derivates of hydroxyethyl starch or other polysaccharides which specifically make it possible, avoiding the previously described disadvantages, to couple to proteins or other pharmaceutical active ingredients, in purely aqueous systems or else in solvent mixtures with water.

[0009] Surprisingly, it has been found that from hydroxyethyl starches which are selectively oxidized at the reducing end of the chain to aldonic acids, as well as from other polysaccharides, such as for example waxy corn starch degradation fractions, acidic alcohols such as N-hydroxysuccinimide could be manufactured to the corresponding ester in dry aprotic solvent, such as dimethylacetamide (DMA) or dimethylformamide (DMF). Such esters can be regarded as activated esters. With nucleophilic NH_2 groups they react to more stable amides in aqueous media. Saponification with water to the free acid and the free alcohol occurs as a side reaction.

[0010] The reaction with HES aldonic acids, for example with N-hydroxysuccinimide succeeds smoothly with EDC in dry DMA with exclusion of water at room temperature to HES acid N- hydroxysuccinimide ester. In this context, it is particularly surprising that no side reactions of the HES molecules occur via reaction of the OH groups of the anhydroglucoses present in extreme excess with EDC as well as that rearrangement reactions of the primarily formed O-acyl isourea of EDC and the aldonic acid to the corresponding N-acyl urea are suppressed.

[0011] The HES aldonic acid esters may be precipitated from solutions in DMA with dry ethanol, isopropanol or acetone, and may be purified by multifold repetition of said process. Such HES acid esters, isolated in substance, may be used for HESylation. In doing so, no side reactions as described above for EDC activated acids occur.

[0012] Further suitable acidic alcohols for preparing the HES or the HES acid aldonic acid esters are detailed in the literature. (V. H. L. Lee, Ed., Peptide and Protein Drug Delivery, Marcel Dekker, 1991, p. 65).

[0013] Advantageously, for example, sulfo-N-hydroxysuccinimide can be used, or phenol derivates. Likewise, N-hydroxybenzotriazole may be used advantageously as alcohol component.

[0014] Suitable hydroxyethyl starch fractions which are selectively oxidized at the reducing end of the chain to aldonic acids in accordance with the state of the art may be used as aldonic acid component. But other starch derivates, such as hydroxypropyl starch, may be used, too. Likewise, hyper-branched starch fractions described in German patent application 102 17 994 may be considered.

Examples

Example 1

Preparation of HES 10/0.4-Acid Esters with N-hydroxysuccinimide

[0015] 5 g of dry hydroxyethyl starch with an average molecular weight Mw=10 000 Dalton and a substitution level MS=0.4, which has been selectively oxidized at the terminal reducing end of the chain in accordance with DE 196 28 705, are dissolved in 30 ml of dry dimethylacetamide at 40°C and, after cooling of the solution, 10 times the molar amounts of N-hydroxysuccinimide are added with exclusion of moisture. The amount of EDC equimolar to the HES acid is then added in portions, and the reaction mixture is allowed to react to completion 24 hours after the addition. The reaction product is subsequently precipitated with dry acetone and purified by repeated reprecipitation.

Example 2

Preparation of Hes 10/0.4-Acid Coupled Myoglobin

[0016] 15 mg of myoglobin are dissolved in 20 ml of distilled water, and the pH is adjusted to 7.5 with sodium hydroxide solution. 1.5 g of HES 10/0.4-acid N-hydroxysuccinimide, prepared as in Example 1, are added in portions to the solution over the course of 1 hour, and the pH is kept constant at 7.5 by adding sodium hydroxide solution.

[0017] The mixture is left to stir overnight.

[0018] The formation of hesylated myoglobin is determined by gel permeation chromatography with a yield of 70 % based on the myoglobin employed.

Claims:

1. An aldonic acid ester of starch fraction or starch fraction derivates which are selectively oxidized at the reducing end of the chain to aldonic acids.

2. The aldonic acid ester as claimed in Claim 1, wherein the starch fractions are amylopectin degradation fractions.

3. The aldonic acid ester as claimed in Claim 2, wherein the amylopectin degradation fractions are obtained by acid degradation and/or degradation by α-amylase of waxy corn starch.

4. The aldonic acid ester as claimed in Claim 3, wherein the starch fractions have an average molecular weight Mw of 2 000-50 000 Dalton and an average branching of 5-15 mol % α-1,6-glycosidic linkages.

5. The aldonic acid ester as claimed in Claim 1, wherein the starch fraction derivatives are hydroxyethyl derivatives of waxy corn starch degradation fractions.

6. The aldonic ester as claimed in Claim 5, wherein the average molecular weight Mw of the hydroxyethyl starch fractions is in the range of 2-300 000 Dalton, and the substitution level MS is between 0.1 and 0.8, and the C2/C6 ratio of the substituents on carbon atoms C2 and C6 of the anhydroglucoses is between 2 and 15.

7. The aldonic acid ester as claimed in Claims 1-6, wherein the alcohol component is N-hydroxysuccinimide, sulfo-N-hydroxysuccinimide, substituted phenols or hydroxybenzotriazole.

8. The aldonic acid ester as claimed in Claims 1-6, wherein the alcohol component is N-hydroxysuccinimide and sulfo-N-hydroxysuccinimide.

9. A method for preparing aldonic acid ester as claimed in Claims 1-8, wherein anhydrous aldonic acids or aldonic acid lactons, respectively, are dissolved in anhydrous aprotic solvent, such as dimethyl sulfoxide (DMSO), N-methylpyrrolidone, dimethylacetamide (DMA) or dimethylformamide (DMF), optionally under heat, 5 to 50-fold molar excess of the alcohol component are added to the reaction mixture and then 1 to 3-fold molar excess, based on the aldonic acid, of EDC (1-ethyl-3-(3-dimethyl-aminopropy1)carbodiimide) are added in portion and then the reaction mixture is allowed to react to completion 24 hours at room temperature.

10. A method for preparing pharmaceutical active ingredients coupled to polysaccharides or polysaccharide derivatives on free amino functions, wherein esters of polysaccharides or polysaccharide derivates which are selectively oxidized at the reducing end of the chain to aldonic acids are reacted therewith resulting in the formation of stable amide linkages.

11. The method as claimed in Claim 10, wherein the esters are esters of N-hydroxysuccinimide, sulfo-N-hydroxysuccinimide, substituted phenols or hydroxybenzotriazole.

12. The method as claimed in Claim 11, wherein the esters are esters of hydroxysuccinimide and sulfo-N-hydroxysuccinimide.

13. The method as claimed in Claims 10, 11 and 12, wherein the polysaccharides are waxy corn starch degradation fractions and hydroxyethyl derivatives of waxy corn starch degradation fractions.

Summary

Disadvantages in the form of undesired side reactions occur in the coupling of polysaccharide derivates, such as hydroxyethyl starch (HES) to pharmaceutical active ingredients in aqueous media. A new method for coupling of polysaccharide derivates to pharmaceutical active ingredients in aqueous media shall be found, which avoids said disadvantages.

The object is solved by the provision of new polysaccharide aldonic acid esters, which allow for coupling of the polysaccharide aldonic acid esters to pharmaceutical active ingredients without said disadvantages.

Improved coupling method of polysaccharide aldonic acids to pharmaceutical active ingredients in aqueous media.